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Expression of the calcium binding proteins Necab-1, -2 and -3 in the adult mouse hippocampus and dentate gyrus

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The family of EF-hand calcium binding proteins is composed of more than 250 members. In search for other neuronal markers, we studied the expression pattern of Necab-1, -2 and -3 in the Ammons horn of adult mice at the gene- and protein levels using in-situ hybridization and immunohistochemistry. The genes for the three Necab's were expressed in specific, non-overlapping areas of the hippocampus. A minority of the Necab-positive interneurons were GABA-ergic, and they virtually never coexpressed one of the classical calcium binding proteins (calretinin, calbindin D-28k and parvalbumin). Necab's are promising new neuronal markers in the brain.

1. Introduction

The EF-hand family Ca^{2+}-binding proteins parvalbumin, calbindin D-28k and calretinin are classic phenotypic markers of terminally differentiated, non-overlapping subclasses of neurons. Secretagogin (Mulder et al., 2009) is a recent addition to the field of these useful tags of neuronal subpopulations. These markers are favorite tools in defining cell types,

Abbreviations: ne: not expressed; X: expressed; OB: olfactory bulb; gr: granular layer; gl: glomerular layer; CTX: cortex; Diverse: AON: anterior olfactory nucleus; isl: islands of Calleja; ACB: nucleus accumbens; CP: caudoputamen; TRS: triangular nucleus of septum; OT: olfactory tubercle; TT: tenia tecta (dorsal and ventral parts); LS: lateral septal nucleus; PIR: piriform area; TH: thalamus; PVT: paraventricular nucleus of the thalamus; LP/LD: lateral posterior/dorsal nucleus of the thalamus; CM: central medial nucleus of the thalamus; RH: rhomboid nucleus; MD/IMD: mediodorsal/intermediodorsal nucleus of the thalamus; AD: anterodorsal nucleus of the thalamus; VN: ventral nucleus of the thalamus; HPF: hippocampal formation; HYP: hypothalamus; MM: medial mammillary nucleus; MB: midbrain; SC: superior colliculus; VTA: ventral tegmental area; SN: substantia nigra; RN: red nucleus; DR: dorsal nucleus raphe; MEV: midbrain trigeminal nucleus; P: pons; PB: parabrachial nucleus; PG: pontine gray; TRN: tegmental reticular nucleus; PSV: principal sensory nucleus of the trigeminal; PRN: pontine reticular nucleus; V: motor nucleus of trigeminal; CBX: cerebellar cortex; Pu: Purkinje layer; Gr: granular layer; MY: medulla; VII: facial motor nucleus; XII: hypoglossal nucleus; IO: inferior olivary complex; SPV: spinal nucleus of the trigeminal; LRN: lateral reticular nucleus; MARN: magnocellular reticular nucleus.

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functional systems and targets for diverse pathologies (Schafer and Heizmann, 1996).

The Human Genome Project has revealed that the family of EF-hand calcium binding proteins is much larger than thought. Approximately 250 genes coding for proteins harboring one or more EF-hands have been revealed. A glance at the distribution of the corresponding mRNA's in the Allen-Database suggests that many of these proteins occur in unique patterns of distribution. Therefore, in addition to the classic four "musketeers" (e.g. calbindin D-28k, calretinin, parvalbumin and secretagogin), other members of the family of EF-hand calcium binding proteins could be interesting markers in health and disease.

In a project aiming at locating precisely a large number of these proteins in the brain, we first choose to study the distribution of the neuronal calcium-binding proteins Necab-1, Necab-2 and Necab-3. Only four articles on these proteins exist in the literature, and the first description by Sugita et al. (Sugita et al., 2002) already contained information on the immunohistochemical distribution of Necab-1. According to this study, Necab-1 is expressed mainly in cortical layer 4 pyramidal cells, pyramidal cells of the CA2-region of the hippocampus as well as interneurons therein. In a subpopulation of interneurons, Necab-1 colocalizes with calbindin D-28k in the same cell (Sugita et al., 2002). Comparing these results with the in-situ hybridization images in the Allen-database (http://mouse.brain-map.org/), we remarked certain discrepan cies. The mRNA for Necab-1, for example, does not seem to be expressed in the CA2-region of the hippocampus.

The purpose of the present study was therefore to map the distribution of the three Necabs in the hippocampus of adult mice at the gene- and protein-expression levels using in-situ hybridization and immunohistochemistry. Attention was given to compare their distribution with that of GABA-positive interneurons, particularly those expressing the three classic Ca^{2+}-binding proteins calbindin D-28k, calretinin and parvalbumin.

2. Results

2.1. *General*

We limited our observations to the hippocampus and the hilus of the dentate gyrus. In each case ~100 cells labeled by in-situ hybridization or by immunohistochemistry were counted in adjacent sections to determine the percentage of co-existence.

At the mRNA-level, only the Necab-1 and Necab-2 probes gave satisfactory results, comparable to what expected from the pattern gleaned from the Allen-Database (http://mouse.brain-map.org/). The Necab-3 probe stained with a high background under a multitude of different conditions. The immunohistochemical staining with Necab-2 and Necab-3 antisera gave similar distribution patterns (Table 1).

2.2. *Necab-1*

Cells expressing the mRNA for Necab-1 were scattered throughout the cell-poor layers of the ammons horn and

Table 1 General distribution of the Necab's in the brain.

	OB	CTX	Diverse	TH	HPF	HYP	MB	P	CBX	MY
Necab1	X (gr and gl)	X	AON, isl, ACB, CP, TRS	PVT, LP/LD, CM, RH, MD/IMD	X	Few sparse cells	Sparse cells in: SC, VTA, SN, RN	Few sparse cells	X (mainly Pu) (fewer in Gr)	Sparse cells throughout strong in VII, XII
Necab2	X (strong in gr) (sparse cells in gl)	X (layer1)	AON, ACB, CP, OT strong in TT, LS	Sparse cells strong in PVT	X	Sparse cells strong in MM	Sparse cells in: RN, SC, VTA, DR	Strong in: PB, PG, TRN, PSV few sparse cells	*ne*	Sparse cells strong in IO, SPV, LRN
Necab3	X (gr)	X	PIR	AD, VN	X	*ne*	Sparse cells strong in RN, MEV	Sparse cells in PRN strong in V	X (few cells in Gr)	Sparse cells in: VII, SPV, MARN, IO

the hilus of the dentate gyrus (Fig. 1). No expression of the mRNA for Necab-1 was observed in either the pyramidal-cell layer of CA1-CA3 or the dentate-granule-cell layer.

Necab-1 mRNA, co-existed with GAD-1 mRNA in some neurons (less than 10%) (Fig. 1A and B). No co-existence with calretinin (Fig. 1F), calbindin D-28k or parvalbumin (not shown), was observed. Necab1-positive interneurons were more frequently found than Necab-2-postive ones.

2.3. *Necab-2*

The mRNA for Necab-2 was highly enriched in the pyramidal-cell layer of the CA2 region (Fig. 2A and C) and in some rare interneurons in the rest of the hippocampus.

The immunohistochemical analysis revealed this pattern of distribution to be imitated at the protein level (Fig. 3). No evidence for the co-existence of Necab-2 with calretinin (not shown), calbindin D-28k or parvalbumin (Fig. 3A, D and F) at the mRNA or protein level was found.

2.4. *Necab-3*

The mRNA for Necab-3 was expressed everywhere in the hippocampus, with a slight preferences for certain cells (e.g. dentate gyrus and some interneurons, Fig. 4B).

The Necab-3 antiserum stained the molecular layer of the dentate gyrus and the CA2 zone and rare interneurons. The Necab-3-positive interneurones did not co-express calretinin, calbindin D-28k or parvalbumin (Fig. 4).

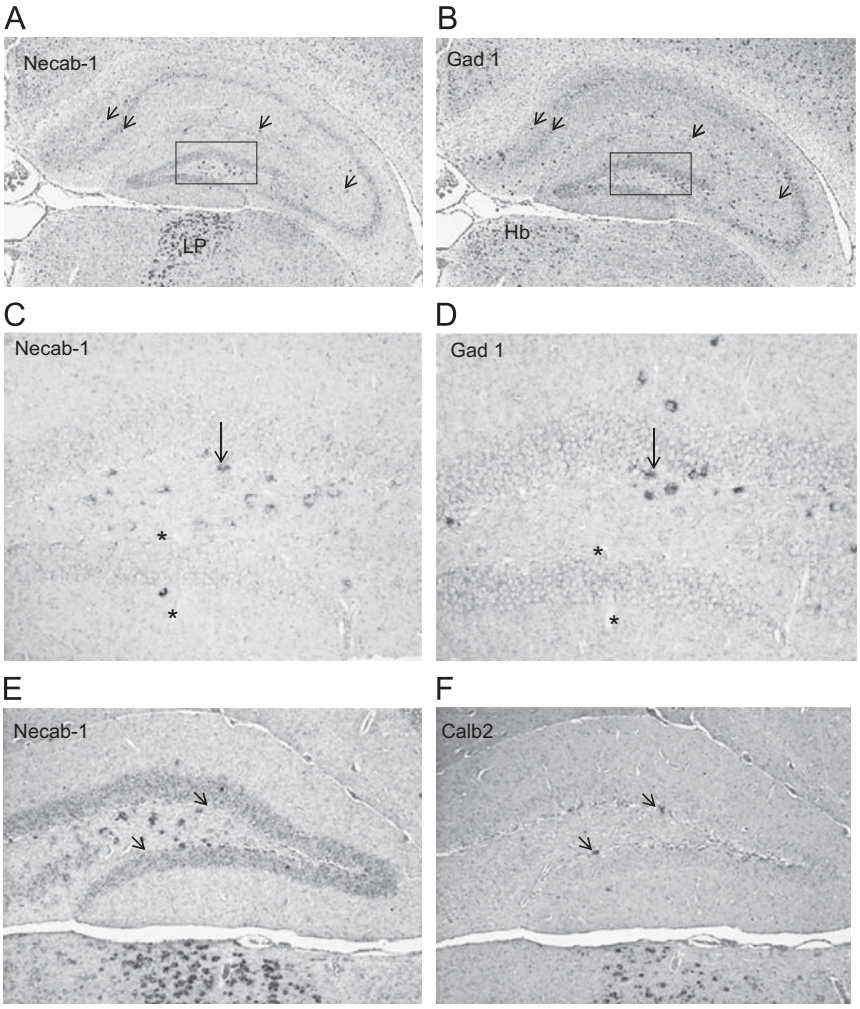

Fig. 1 – (A and B) In-situ hybridization of mRNA for Necab-1 and Gad1: Necab-1 positive cells are located within the *stratum lacunosum moleculare*, the *stratum radiatum and the stratum oriens* of the hippocampus, as well as in the hilus of the dentate gyrus. Only a few (~10%) of the scattered cells expressing the mRNA for Necab-1 (arrows) also express GAD-mRNA. Neurons within the nucleus of the thalamus (LP) express high levels of the mRNA for Necab 1, whereas GADmRNA is rich in the habenular nuclei (Hb). (C and D) Higher magnification of A, respectively to the other section flanking the section depicted in A. Only one out of the approximately 20 cells in this field of view expresses the mRNA's for Necab-1 and GAD1. (E and F) Necab-1 and Calb1 mRNAs are not expressed in the same cells of the hippocampus (arrows), but may co-exist in thalamic projection neurons.

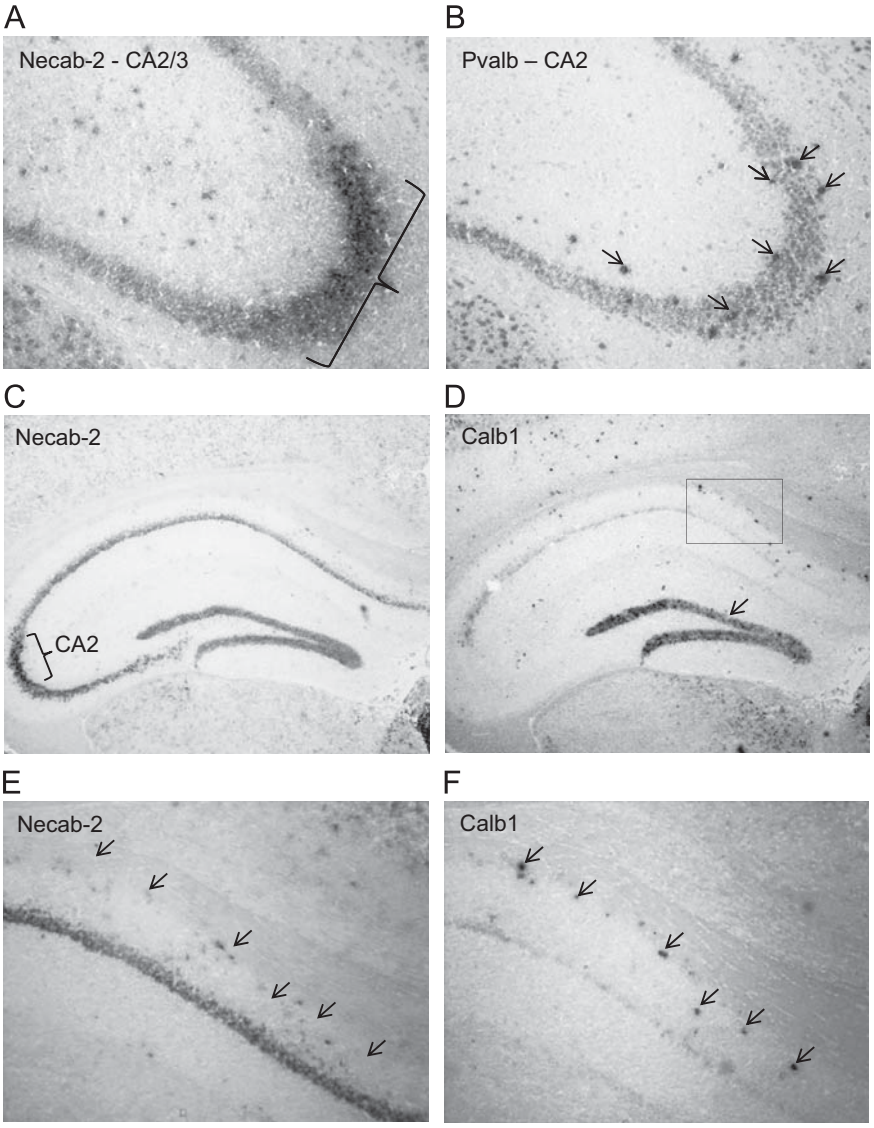

Fig. 2 – In-situ hybridization for Necab-2 mRNA (A, C and E) compared to the distribution of the mRNA for parvalbumin (Pvalb; B) and calbindin D-28k (Calb1, D and F). (A–C) The mRNA for Necab 2 is highly expressed within pyramidal cells of the CA2 region (brackets in A and C). A scattering of positively-stained cells is apparent In the Pvalb-section (arrows in B). (D–F) Calb1 mRNA is enriched in the dentate gyrus (arrow in D) and occurs in scattered neurons in various layers of the hippocampus. Coexistence with Necab1 was never observed (E and F).

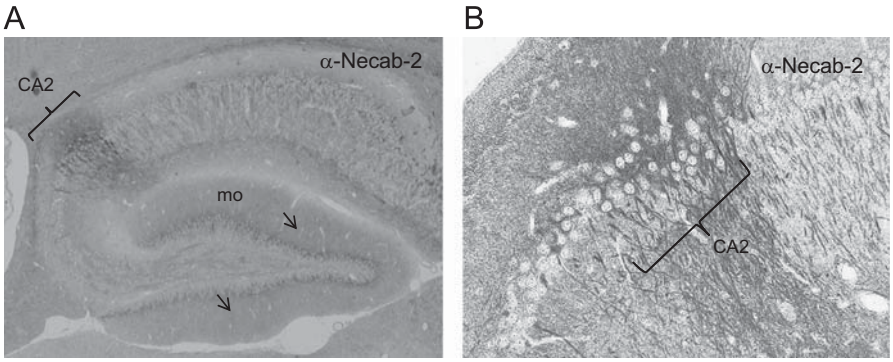

Fig. 3 – (A) Immunohistochemical revelation of the Necab-2 protein. Positive reactivity is observed within pyramidal cells of the CA2 region (bracket). The molecular layer of the dentate gyrus (mo) is also stained (arrows). (B) Higher magnification of A. Positive reaction within the dendrites of pyramidal cells in the CA2 region.

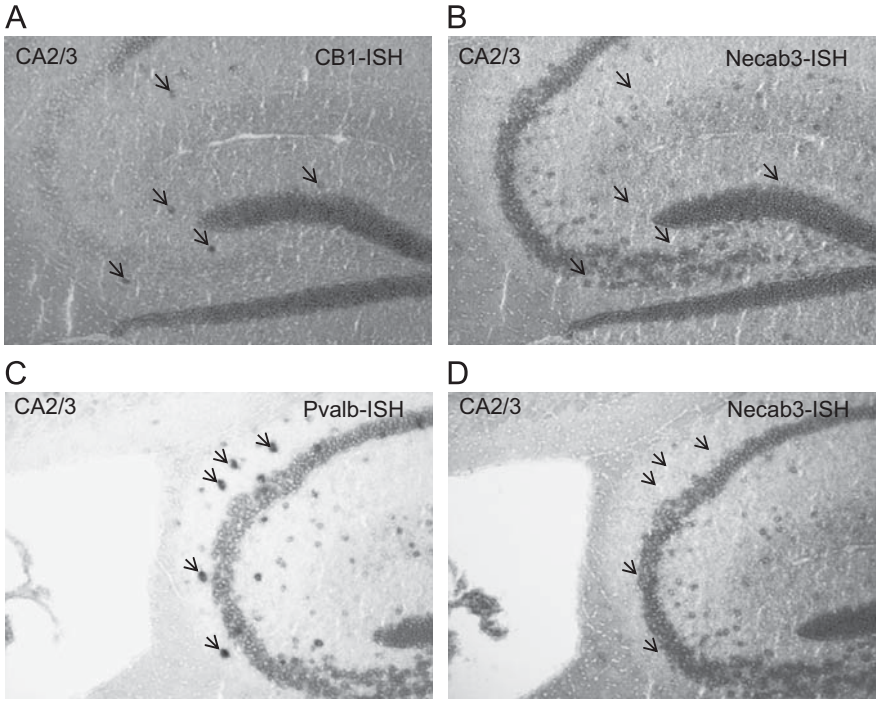

Fig. 4 – In-situ hybridization for Necab-3 mRNA (B and D) compared to the distribution of the mRNA for calbindin D-28k (Calb1, A) and parvalbumin (Pvalb; C). Interneurons positive for parvalbumin or calbindin D-28k (A and C) never express the mRNA for Necab3 (B and D).

3. Discussion

In-situ hybridization revealed a restricted and, for the most part, non-overlapping expression of the mRNAs for Necab-1 and Necab-2 in the hippocampus of adult mice, whereas the results for Necab-3 were difficult to categorize. In the Allen-Database, Necab-3 is shown to be expressed in the granular layer of the dentate gyrus and in the hippocampal CA3 zone.

Necab 1 has been shown by affinity chromatography to interact with the C2-domain of synaptotagmin—a synaptic-vesicle protein that is involved in calcium-dependent exocytosis (Canela et al., 2009). Our data pertaining to the in-situ hybridization of Necab 1 are at variance with previously-reported immunohistochemical findings (Sugita et al., 2002) revealing intense immunoreactivity within pyramidal cells of the CA2 region. We observed mRNA for Necab 1 only within interneurons - as is reported in the Allen-Database - and never in the CA2 region. We presume that in the earlier study, the antibody against Necab-1 cross-reacted with the Necab-2 protein that is expressed within the pyramidal cells of the CA2 region (see our Fig. 2B and C). This supposition is supported by the observation that in the earlier study, immunoblotting experiments revealed the antibody against Necab 1 to cross-react with Necab-2 and Necab-3 proteins in Cos cells that had been transfected with vectors for their expression (Sugita et al., 2002).

Necab-2 has been reported to be a downstream target of Pax6—a transcription factor involved in the development of the eye and the brain. In the development of the retina, it forms part of a signal-transduction pathway (Bernier et al., 2001). Necab-2 also interacts with the adenosine A2-receptor and the glutamate-5 receptor in a calcium dependent manner (Canela et al., 2007; Canela et al., 2009). In both cases, this interaction leads to a downregulation of the cell-surface receptors and a modulation of their activities (Canela et al., 2007; Canela et al., 2009). Our data of in-situ hybridization and immunohistochemistry of Necab-2 confirm the staining in the basal ganglia as published by others (Canela et al., 2007), but not the reaction of the CA1 pyramidal cells (Canela et al., 2009). In the hippocampus, only the CA2 pyramidal cells and scattered cells expressed the Necab 2 gene, consistent with results of the Allen Database.

Necab 3 may be a substrate of Nek2 and play thus a role in the Golgi apparatus (Yoo et al., 2004), whereas other task in connection with β-amyloid (Yoo et al., 2004) could not be confirmed (Sugita et al., 2002). The hybridization in the granule cells of the dentate gyrus and the pyramidal cells of the CA3 region, as visible in the Allen-Database, was difficult to reproduce.

The antiserum against Necab-2 highlights the CA2 pyramidal cells, but also stains dentate granule cells and some scattered interneurons. This pattern is similar to that of the Necab-3 antiserum. Being directed against recombinant proteins which share ~50% sequence homologies, they probably cross-react with the other two members of this family. Therefore, immunohistochemical studies of the distribution of Necab's in the brain may be less reliable than in-situ hybridization experiments.

4. Experimental Procedures

4.1. *In-situ hybridization*

The brains were dissected from 7 deeply anaesthetized Ketalar [(Parke-Davis) 75 mg/kg of body weight] and Xylazine [(Streuli)

10 mg/kg of body weight], adult female and male mice (C57 BI/6) and immediately frozen on dry ice. They were then stored at −70 °C until use. Cryosections were prepared from the frozen brains. In-situ hybridization was performed as recommended by Roche (*DIG application manual for nonradioactive* in-situ *hybridization, Roche Applied Science, 3rd Edition*). To improve the specificity of the gene-expression patterns, several of the parameters were modified (incubation temperature: 60 °C, incubation time: 16 h/concentration of the RNA probe: 1/100, concentration of proteinase K: 2 μl/ml). The digoxigenin-labeled RNA-probes were prepared following the polymerase-chain-reaction-based strategy (http://www.brain-map.org). The RNA of adult-mouse brains (Zyagen) was reverse transcribed using a commercial kit (Applied Biosystems). The polymerase chain-reaction was performed under standard conditions. The primers used to generate specific ISH probes for each of the genes tested were the same as those described in the Allen Brain Atlas Database (http://www.brain-map.org):

Necab-1 (probe size: 605 bp):
Forward: GACTGAATGTTAGGATCTGGGC
Reverse: AGCACCTGACCAGGACTATGAT
Necab-2 (probe size: 617 bp):
Forward: TACCATCGATTCAGACAACACC
Reverse: AGGTACTGTCTCAGGGAATCCA
Necab-3 (probe size: 693 bp):
Forward: GCTGCTACATGAACTTCACAGG
Reverse: TAGACTGAGTTGGCCTAGCCTC
Gad1 (probe size: 982 bp):
Forward: TGTGCCCAAACTGGTCCT
Reverse: TGGCCGATGATTCTGGTT
Calb1 (probe size: 579 bp):
Forward: GAACTATTCAGGATGTGTGGCA
Reverse: GGGCTATGGTCATACTCTCTGG
Calb2 (probe size: 893 bp):
Forward: GATGCTGACGGAAATGGG
Reverse: CCCTACCAGCCACCCTCT
Pvalb (probe size: 823 bp):
Forward: TCTGCTCATCCAAGTTGCAG
Reverse: TCCTGAAGGACTCAACCCC

The forward and reverse primers were flanked by T3- and SP6-RNA polymerase core-promoter sequences, respectively (T3: AATTAACCCTCACTAAAGG; SP6: GCGATTTAGGTGACAC TATAG).

In addition, 2 mice were deeply anesthetized, and perfused transcardially with 0.9% NaCl, followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, postfixed overnight and embedded in paraffin. 3 μm thin sections were incubated with the same probes as above and revealed with the Ventana-Discovery system. The procedure involved using alkaline-phosphatase labeled anti-DIG. Details of the method have been recently published (Girard et al., 2011). Different probes were incubated with adjacent sections, so that the gene of interest was flanked by two identical control probes: for example: Gad1/Necab-1/Gad-1 or Pvalb/Necab-2/Pvalb, etc.

4.2. *Immunohistochemistry*

A polyclonal antibody against Necab 2 was kindly provided by Dr. Francisco Ciruela (University of Barcelona, Spain) (Canela et al., 2007) and one against Necab 3 by Dr. Kunsoo Rhee, South Korea (Yoo et al., 2004).

Polyclonal antibodies against calbindin D-28k, parvalbumin and calretinin were received from Swant (Marly, Switzerland). These antibodies and the immunhistochemical technique have been described previously (Gerig and Celio 2007; Meszar et al., 2012).

5. Conclusion

The three Necab-proteins appear to be involved in diverse activities, including the calcium-dependent activation of target proteins (Canela et al., 2007; Canela et al., 2009), calcium-buffering (Canela et al., 2009; Sugita et al., 2002) and self-regulation via calcium binding (Canela et al., 2007; Canela et al., 2009). The finding that each of the genes for the three Necab-isoforms are expressed in specific regions of the hippocampus, and that together their expression embraced most of its expanse, indicates that notwithstanding their structural similarities, the three calcium-binding proteins might have different functional roles in this part of the brain.

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